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ORIGINAL ARTICLE

Relationships between Podolic cattle breeds assessed by single nucleotide polymorphisms (SNPs) genotyping

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Summary

Italian Maremmana, Turkish Grey and Hungarian Grey breeds belong to the same Podolic group of cattle, have a similar conformation and recently experienced a similar demographic reduction. The aim of this study was to assess the relationship among the analysed Podolic breeds and to verify whether their genetic state reflects their history. To do so, approximately 100 single nucleotide polymorphisms (SNPs) were genotyped on individuals belonging to these breeds and compared to genotypes of individuals of two Italian beef breeds, Marchigiana and Piemontese, which underwent different selection and migration histories. Population genetic parameters such as allelic frequencies and heterozygosity values were assessed, genetic distances calculated and assignment test performed to evaluate the possibility of recent admixture between the populations. The data show that the physical similarity among the Podolic breeds examined, and particularly between Hungarian Grey and Maremmana cattle that experienced admixture in the recent past, is mainly morphological. The assignment of individuals from genotype data was achieved using Bayesian inference, confirming that the set of chosen SNPs is able to distinguish among the breeds and that the breeds are genetically distinct. Individuals of Turkish Grey breed were clearly assigned to their breed of origin for all clustering alternatives, showing that this breed can be differentiated from the others on the basis of the allelic frequencies. Remarkably, in the Turkish Grey there were differences observed between the population of Enez district, where *in situ* conservation studies are practised, and that of Bandirma district of Balikesir, where *ex situ* conservation studies are practised out of the original raising area. In conclusion, this study demonstrates that molecular data could be used to reveal an unbiased view of past events and provide the basis for a rational exploitation of livestock, suggesting appropriate cross-breeding plans based on genetic distance or breeding strategies that include the population structure.

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Introduction

Over the past 15 years, 300 out of 6000 breeds of all farm animal species identified by FAO have gone extinct (Scherf 2000). It is argued in the World Watch List for Domestic Animal Diversity (DAD) that 1350 farm animal breeds currently face extinction. The danger of extinction of farm animal breeds is mainly attributable to three factors: the first is genetic erosion because of artificial insemination. The second factor is strong economic pressure on the farmer to focus on single traits, such as milk production. The third factor is unrestricted and indiscriminate cross-breeding, especially in developing countries (Soysal *et al.* 2004).

Podolic cattle include a very ancient group of breeds, considered to be straight descendant from the Auroch (*Bos primigenius*). Podolic breeds are present in various European areas, and many of them are seriously endangered of extinction.

Hungarian Grey, Maremmana and Turkish Grey belong to the same Podolic group of cattle and show similar external conformation. These breeds recently underwent a similar demographic reduction. All three of these breeds face risks for their future survival because of inbreeding, indiscriminate cross-breeding and substitution with cosmopolitan more productive breeds.

The breeds

Maremmana

The breed can be traced back to Grey Steppe cattle which entered Italy in large numbers during the 14th to 18th centuries. Herdbook registration started in 1935 (total breed population 274 000 head). Since 1945, head numbers have declined dramatically because of the changes in land use and mechanization, and by the mid-1960s extinction was predicted. The breed recovered between 1965 and 1975 because of its ability to adapt to the environmental constraints of the hilly areas of the Maremma, reaching 60 000 head in 1975. In 1992, 10 000 head, of which 4000 females and 120 sires, were registered in the herdbook. Since then the number remained constant.

Maróti-Agóts *et al.* (2005) report that the Maremmana cows are significantly bigger in every body measurement than Hungarian Grey cattle, and in particular there are significant differences of rump length/body length index.

Hungarian Grey

The breed was imported by the Hungarian conquerors who came from the Podolic area in the south, in the 9th century. There is evidence of the presence of similar cattle in Egypt and Italy. Explicit reference to long-horned, 'magnus cornutes boves Hungaricos', first appears in a 16th century document. The breed became common by the 17–18th century.

A radical decline followed World War II and the breed nearly became extinct in 1947–1967. During the late 1950s, 1800 of the 2000–3000 cows were mated with sires of the Kostroma dairy breed. By 1962 only 200 purebred Hungarian Grey cows and six bulls were saved (Bodó *et al.* 1996; Bartosiewicz 1997). Inbreeding was avoided by using a rotational mating scheme based on six local Hugarian Grey sires, two imported sires of the same breed and three Maremmana sires introduced during the early 1970s. After 7–9 generations, the initial lines became completely randomized.

Turkish Grey

Found in north-western Turkey, this breed is a tri-purpose breed: kept for milk and meat as well as being used as a work animal. The breed came from the Grey Steppe type and originated from the Iskar breed of Bulgaria (Mason 1996). It is believed that Turkish Grey cattle are the ancestor or relatives of European Grey cattle found in Italy, Bulgaria and Hungary. The breed has a 'postendangered' status; however, efforts are still required to maintain the grey cattle population. Native grey cattle represent the most interesting cattle breed in Turkey, especially because of its resistance to parasites and its ability of using wetland pasture, and as a consequence its sustainable use of marginal lands (Soysal & Kok 2006).

The aim of this study was to assess the relationship between Hungarian Grey, Maremmana and Turkish Grey. To do so, single nucleotide polymorphisms (SNPs) were genotyped on individuals belonging to the three Podolic breeds as well as on individuals of two Italian beef breeds, Marchigiana and Piemontese, which have different selection and migration histories, and were chosen as example of unrelated breeds.

SNPs are abundant in the genome; genotyping results are easy to reproduce in different laboratories and are simple to score. The usefulness of SNPs in population genetics has been demonstrated in several studies on the last decades, and they have been recently used to discovering signatures of selection (Akey *et al.* 2002; Kelley *et al.* 2006; Luikart *et al.* 2003; Pariset *et al.* 2006a; Pariset *et al.* 2009) and in evaluating population structure (Pariset *et al.* 2006b; Negrini *et al.* 2008).

Materials and methods

Samples

Animals were sampled from their native regions: the Hungarian Grey (HU) population in Hortobagy (Hungary), the Maremmana (MM) from the population of Castelporziano (Rome), and the Turkish Grey (GS) in the Enez district of the Edirne Province of Trakya and in the Bandirma Province of Balikesir (Turkey). Sixty-three (HU), 93 (MM) and 93 (GS) individuals were sampled in each population; DNA from blood samples was extracted using Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA) following the manufacturer instructions. Samples and data of Piemontese (PD) and Marchigiana (MR) cattle were obtained from a previous project (EU-GemQual QLRT CT2000-0147).

SNPs genotyping

Ninety-nine SNPs, selected from a panel of 701 SNPs in candidate genes for meat quality previously characterized (Williams *et al.* 2009), were genotyped on individuals belonging to the five breeds. SNPs were discovered by sequencing a panel of eight individuals each from a different breed.

Genotyping was performed by outsourcing to Kbiosciences (http://www.Kbioscience.co.uk), using quality control criteria as negative controls, interplate and intraplate duplicate testing of a known DNA. Generally, a genotyping repeatability >99% was achieved.

Statistical analysis

Allele frequencies were calculated using *Fstat* 2.93 (Goudet 1995, 2001). *Fis*, Ho and He were estimated for each locus using *Powermarker* (Liu & Muse 2001). The same software was used to test for deviation from Hardy–Weinberg equilibrium (HWE) for each locus and population and for loci over all populations using a Markov chain of 100 000 steps and 1000 dememorization steps. GENEPOP version 4.0 (Raymond & Rousset 1995) was used to calculate *F* statistics per breed, following Weir & Cockerham (1984) and to characterize genetic differentiation among breeds by estimating overall and pairwise *Fst* values. Nei (1972) genetic distances between populations were calculated using *Powermarker*. The same software was used to infer haplotypes.

To evaluate whether some of the loci supposed neutral in this study could be identified as outliers, and therefore genotyped SNPs were unsuitable to be used as markers, the approach proposed by Beaumont & Nichols (1996), further developed by Beaumont & Balding (2004), and implemented in the *FDIST2* software (http://www.rubic.rdg.ac.uk/~mab/software.html) was used. For each locus, the allele frequencies were used to compute *Fst* values conditional on heterozygosity and to calculate P-values for each locus. Each simulation included five populations, 91 loci and an expected *Fst* of 0.104. Population data sets were built using 50 000 coalescent simulations on real data using the infinite alleles model.

Individuals were clustered by applying a parametric genetic admixture analysis implemented in the *Structure* 2.0 software (Pritchard *et al.* 2000). This software uses a model-based clustering method that employs a Markov Chain to estimate the posterior distribution (q) of the admixture coefficient of each individual, to characterize parental populations, to assign individuals to these populations, to detect admixed individuals and to estimate individual admixture starting from allele frequency. Results were obtained using a burn-in period of 100 000 followed by 200 000 Markov chain Monte Carlo (MCMC) repeats and considering SNPs frequencies independent among populations. A number of genetic clusters (K) ranging from 2 to 6 was tested using the admixture model; four runs for each K were performed. Graphical reconstruction of *Structure* results were produced by using *Distruct* 1.1 (Rosenberg 2004).

Results

SNPs genotyping

Ninety-nine polymorphic SNPs were used to genotype 311 individuals belonging to the five breeds. Of these, 91 SNPs resulted polymorphic and suitable for the analyses. A total of 26 006 genotypes were produced, and the frequencies of the major alleles are reported in Table S1. The SNP data were analysed to assess their neutrality using *Fdist* (Beaumont & Nichols 1996), and all SNPs resulted within the 99% upper and lower limits of distribution. When more than one SNP within the same gene was genotyped, haplotypes have been inferred and used for the subsequent analyses (Table S2), for a final data set of 67 markers. Major allele frequency, Expected and Observed heterozygosity, Polimorfic information content (PIC), *Fis*, p-value relative to HW test and chromosomal location relative to Btau 4.0 for each polymorphic marker are reported in Tables S1 and S2, respectively.

Genetic diversity and differentiation of cattle breeds

Observed heterozygosity of the 67 markers determined from SNP frequencies ranged from 0.557 to 0.017, with a mean of 0.346. Expected heterozygosity ranged from 0.649 to 0.024, with a mean of 0.386. The frequencies of the major alleles ranged from 0.988 to 0.428. Frequency of the minor allele was >5% in all but three SNPs (POMC_b1_63T, VCL_a1_160T, PRKAA2_a1_88C). *Fis* value of the markers ranged from 0.483 to −0.138, with a mean of 0.138. Significant deviations from HWE over all populations (p-value <0.05) were observed in 38 markers.

Within population variance estimate (*Fis*) per population ranged from −0.020 (PD) to 0.186 (GS) (Table 1). Positive *Fis* was observed in Turkish Grey breed. A moderately positive *Fis* value was observed also in Marchigiana breed (Table 1).

The pairwise *Fst* between populations showed a maximum (0.124) between Hungarian Grey and Maremmana, and a minimum (0.081) between Marchigiana and Piemontese (Table 2).

The Nei standard genetic distance (Nei 1972) indicates a maximum distance between Marchigiana and Turkish Grey (0.070) and a minimum distance between Marchigiana and Maremmana (0.051). Considering only the three Podolic breeds, the greater distance is observed between Hungarian Grey and Maremmana (0.066) (Table 3).

Individual assignment

To estimate the number of genetic clusters among the 311 individuals, a parametric genetic mixture analysis implemented in the *Structure* 2.0 software (Pritchard *et al.* 2000) was performed. Between two and six clusters (K values) were tested using the admixture model, assuming that each individual does not necessarily have a genetic background originating from one of the K populations. Consistent results across runs were obtained.

To identify the optimal K value and hence identify the most reliable result we applied the methodology described in Evanno *et al.* (2005), concluding that 5 was the optimal K.

A graphic representation of the estimated membership coefficients to the clusters for each individual, obtained running structure setting K from 2 to 5, is shown in Figure 1. Each individual is represented by a single vertical line broken into K coloured segments, whose lengths are proportional to each of the K inferred clusters.

For K = 5 most of the individuals could be unambiguously assigned to the five breed clusters. Table 4 demonstrates the proportion of membership of the five breeds in each of the five clusters. Individuals of Piemontese and Marchigiana show the highest level of genetic admixture and could not be differentiated for K values between 2 to 5. Differentiation within the Turkish Grey breed is first observed for K = 2, with 0.23% of the individuals assigned to a separate cluster before differences at the breed level are detected. Raising the K value to 5, the Turkish Grey individuals remain grouped in two distinct populations (Figure 1).

Table 1 *F* Statistics per breed over all loci following Weir & Cockerham (1996)

Breed	*Fst*	*Fit*	*Fis*
Turkish Grey	0.3235	0.3975	0.1861
Hungarian Grey	0.3596	0.3431	−0.0480
Maremmana	0.3795	0.3541	−0.0718
Marchigiana	0.3231	0.3617	0.1066
Piemontese	0.3763	0.3688	−0.0202

Table 2 Pairwise *Fst* between breeds estimated as in Weir & Cockerham (1996)

	Turkish Grey	Hungarian Grey	Maremmana	Marchigiana
Hungarian Grey	0.118			
Maremmana	0.107	0.124		
Marchigiana	0.100	0.107	0.081	
Piemontese	0.109	0.119	0.094	0.081

Table 3 Nei (1972) standard diversity index

OTU	Turkish Grey	Hungarian Grey	Maremmana	Marchigiana
Hungarian Grey	0.064			
Maremmana	0.063	0.066		
Marchigiana	0.070	0.059	0.051	
Piemontese	0.062	0.069	0.056	0.057

Discussion

The SNPs used in this study are not a random sample. However, they do not show to be under selection and are fairly scattered among all chromosomes (with exception of 23, 24 and 27; Table S1). A selection bias may be present because of the small size of the animal panel used for SNPs discovery. However, this is a limit common to many studies, even using larger SNP panels (The Bovine HapMap Consortium 2009). Our panel was composed by eight individuals belonging to different breeds which are deemed to

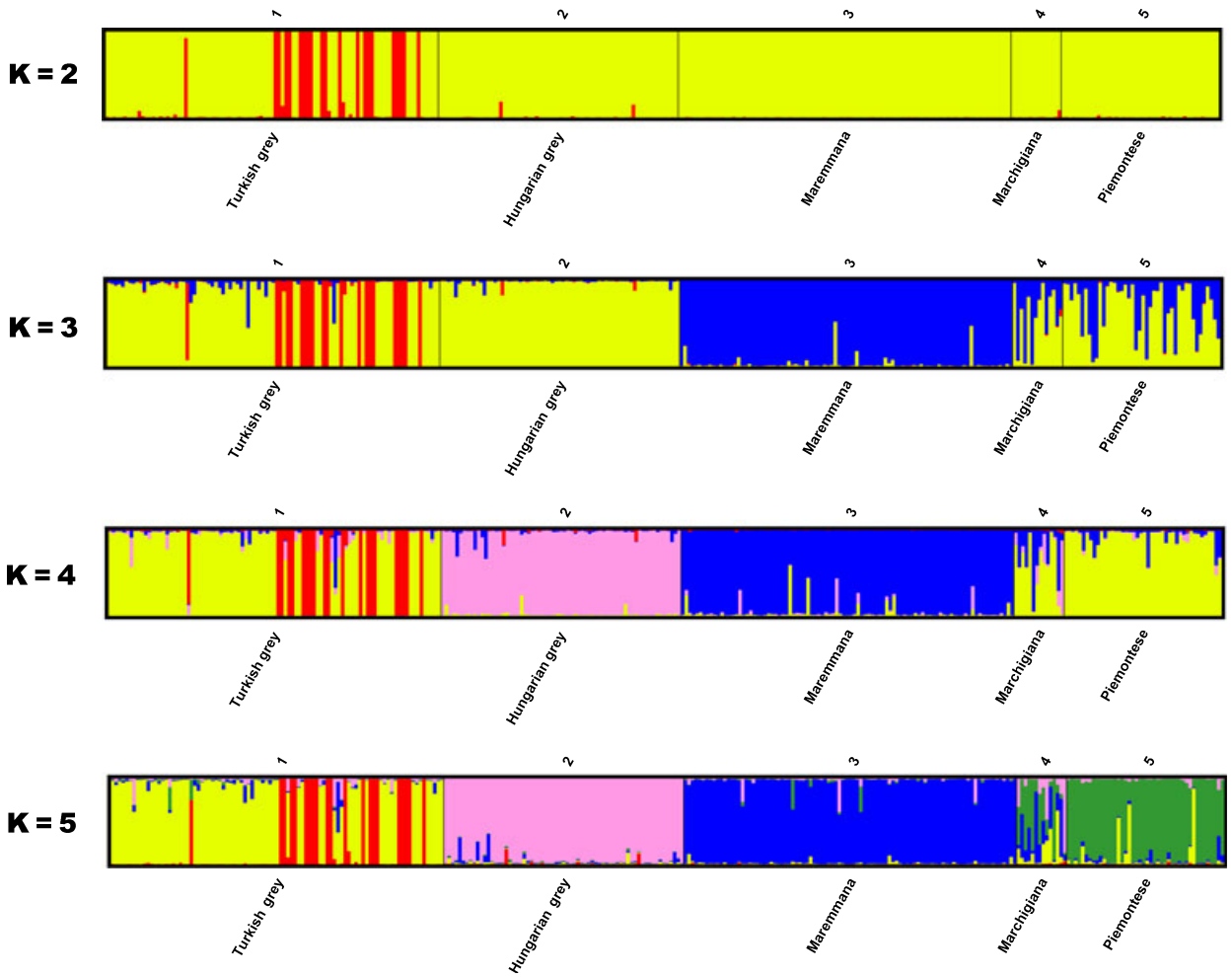

Figure 1 Estimated membership coefficients of each individual to the model-based clusters obtained running structure setting K from 2 to 5. Each individual is represented by a single vertical line broken into K coloured segments, whose lengths are proportional to each of the K inferred clusters. The predefined groups (breeds) are separated by black lines.

Table 4 Proportion of assignment of individuals of the five breeds in each of the five inferred clusters obtained from STRUCTURE analysis

Predefined populations	Inferred clusters				
	1	2	3	4	5
Turkish Grey	0.229	0.706	0.027	0.026	0.012
Hungarian Grey	0.008	0.013	0.947	0.025	0.006
Maremmana	0.003	0.012	0.021	0.949	0.015
Marchigiana	0.008	0.149	0.168	0.283	0.392
Piemontese	0.004	0.074	0.017	0.031	0.873

diverge since many generations (Williams *et al.* 2009) and therefore should include a substantial amount of species variation. Within population variance estimate (*Fis*) was positive in Turkish Grey breed, indicating a significant level of inbreeding. A value suggesting moderate inbreeding is also observed in Marchigiana. The other three breeds showed values compatible with random mating. Therefore we can conclude that mating strategies used in Maremmana and Hungarian Grey during the recovery were appropriate, while a different mating strategy could be suggested at least in one of the Turkish Grey populations.

The estimate of Nei's genetic distances show that Maremmana is more distant from Hungarian Grey (0.66) than from Turkish Grey (0.63), despite the recent admixture with the former breed. Maremmana is closer to Marchigiana (0.51) than to either Hungarian Grey or Turkish Grey. Maximum genetic distances were observed between Turkish Grey and Marchigiana (0.70) and Hungarian Grey and Piemontese (0.62). Data are supported also by pairwise *Fst*, showing that Maremmana is closer to Turkish

Grey (0.107) than to Hungarian Grey (0.124). The high *Fst* values observed in Hungarian Grey (Table 1) may reflect human selection, a long-time isolation of the breed, or a limited number of founders. The last two hypotheses seem more compatible with the history of the breed.

From the *Structure* analysis, already with a K value of 2 about 1/4 of the Turkish Grey individuals were assigned to a separate group, remaining apart from the other breeds. This may be attributable to the high level of inbreeding in the population analysed (*Fis* 0.19). It must be noticed that Turkish Grey cattle samples were collected from two distinct populations: the first raised by breeders of Enez district, the second belonging to the Bandirma district of Balikesir. Raising the K value Maremmana (K = 3), then Hungarian Grey (K = 4) and finally Marchigiana and Piemontese together (K = 5) are assigned to separate groups. It can be hypothesized that the different conservation strategies used in Enez district and in Bandirma district have very different effects on the genetic asset of the populations. Individuals of Piemontese and Marchigiana show the highest level of genetic admixture, revealing that the genotype distributions of these two breeds are more similar than those of the other breeds examined. This is not surprising, in that the two breeds, beside having a different history, underwent specific selection for beef production.

From this analysis, despite their similar morphology, Hungarian Grey and Maremmana are clearly identified as genetically distinct breeds. This could be attributed to either a different origin of the breeds or a consequence of the recent history, that led to the selection and probably fixation of genes. The two breeds were found differentiated on the basis of their allelic frequencies in a previous study (Valentini *et al.* 2006), and this confirms also the morphological differences reported by Maróti-Agóts *et al.* (2005).

As for the Turkish Grey, we observed interesting differences between the population raised by breeders of Enez district, where *in situ* conservation studies are practised, and belonging to the Bandirma district of Balikesir, where *ex situ* conservation studies are practised 400 km far away from the original grey cattle raising area, in the Agriculture Research farm of Ministry of Agriculture. Turkey is very close to cattle domestication centre (Edwards *et al.* 2007); therefore, a higher differentiation is expected because the time for drift and distance to domestication bottleneck and this could explain why one of the two population results genetically differentiated for any value of K tested. Besides being raised far from the original raising area of Grey cattle, the *ex-situ* herds of Bandirma are subject to legal and financial limitation, i.e. farmers are not able to buy new members, and this leads to increment of inbreeding and to genetic drift. On the contrary, private farmers, raising the *in situ* herds for conservation purposes, avoid inbreeding because of more flexibility in management decisions. Moreover, the Enez herds were established 5 years after those of Bandirma, and this could represent another source of difference.

In conclusion, this study demonstrates that morphology and anecdotic accounts might be deceiving in describing a population, as it was in the case of Maremmana and Hungarian Grey. Molecular data are very suggestive, and the observed genetic differentiation of the breeds may deserve more investigation. Further analysis could help in tracing an unbiased picture of past events and provide the basis for a rational exploitation of livestock, suggesting appropriate cross-breeding plans based on genetic distance, or breeding strategies that include the population structure.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Major Allele frequency, Expected and Observed Heterozygosity, Polimorfic information content (PIC), *Fis* (*f*) values for the genotyped SNPs, Exact p-value in HW test of Guo and Thompson (1992). Chromosome (BTA) and chromosomal location relative to Btau 4.0 genome sequence are those reported by Williams *et al*. (2009).

Table S2 SNPs used for haplotype reconstruction (SNPs), Major Allele frequency, Expected and Observed Heterozygosity, Polymorphic information content (PIC), *Fis* (*f*), Exact p-value in HW test of Guo and Thompson (1992).

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